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Investigation of the presence of the virus in the urine and fecal samples of COVID-19 diagnosed and suspected patients by RT-PCR method: A different perspective on fecal-oral transmission theory

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Abstract

With the sudden COVID-19 outbreak in 2019 which has taken over the whole world, drastic changes have taken place on a global level from the understanding of hygiene to living conditions. Although the disease is often detected using respiratory tract samples with PCR tests, many publications state that the virus can also be traced in various body wastes. In our study, we aimed to determine the frequency of the virus in 50 urine and 50 stool samples and to reveal the relationship between the patients' stool PCR results according to Computed tomography (CT), biochemical tests, and the positivity of respiratory PCR test with microbiological samples. The presence of SARS-CoV2 RNA in the urine and stool samples of the patients was detected by qRT-PCR methods. Shapiro-Wilk normality test was applied to the CT, respiratory tract PCR and biochemistry results of the patients. As shown by the results, while none of the 50 patients had positive urine samples, we detected COVID-19 viral shedding in the stool specimens of 15 patients. Respiratory PCR test was negative in 4 of the stool-positive patients. No significant correlation was found between CT results and the biochemistry results of 15 patients with positive stools. In our study, the rate of viral shedding in feces was found to be 30%, which we think may be a finding to demonstrate how the pandemic rapidly progresses in cities due to the viability of the virus outside the body.

Keywords: COVID-19, stool, urine, RT-PCR methods, radiology findings

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, which emerged in China in 2019 and spread worldwide, was named COVID-19 and infected a total of 603,711,760 people worldwide, with the death toll currently standing at 6,484,136 deaths [1]. In Türkiye, where the first case was seen on March 11, 2020, a total of 16,797,750 diagnosed cases and 100,840 deaths have been recorded so far [2].

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While microbiological and radiological examinations are used in the diagnosis of COVID-19, biochemical tests and parameters such as disease risk grading, patient's age, and sanitation conditions are known to be used in the follow-up of the infection [3]. Although not specific to the disease, the symptoms often manifest with a sore throat, muscle pain, fever, cough, headache, weakness, and shortness of breath. Again, the disease may progress asymptomatically in some patients. Mortality is observed in some patients due to the rapid progression of the disease to severe pneumonia [4-6].

Although the real-time polymerase chain reaction (RT-PCR) test, which is performed with a throat swab sample, is used as the gold standard worldwide for the diagnosis of COVID-19, Computed tomography (CT), despite not being classified as a screening test, has been used extensively in patients with negative PCR tests but clinical symptoms due to the lengthy delivery at the beginning of the pandemic and long turnaround time of PCR tests, as well as false negatives especially in the early period [6-8]. Again, some researchers have developed a scale called “corona score” in which they use factors like age and gender as well as biochemical parameters such as C-Reactive Protein (CRP), ferritin, lactate dehydrogenase (LDH), lymphocyte count, neutrophil count, and CT results in the diagnosis of COVID-19 positive and negative patients [9].

Of patients who applied to the emergency department with the suspicion of COVID-19, low albumin levels were observed in 76%, elevated CRP in 58%, and high LDH in 57%, and routine biochemical tests were performed for these patients along with these parameters [10]. COVID-19, which is transmitted primarily by droplets and is a respiratory disease, can also be isolated from fecal and urine samples, albeit rarely. Studies have shown that isolation from urine samples is around 1%, whereas isolation from fecal samples is 29% [11-14].

Again, in the study by Xu et al., a pediatric patient with preliminary signs of COVID-19 tested negative in the PCR test using a nasopharyngeal swab specimen; however, the researchers obtained a positive result when they repeated PCR using a rectal swab specimen from the same patient. The study team then studied RT-PCR by taking rectal samples from 8 other patients who showed symptoms of the disease but had negative nasopharyngeal samples, they determined positive rectal samples in these patients and strengthened the argument that rectal samples can also be taken from patients whose nasopharyngeal swabs could not be found to be positive [15]. SARS-CoV-2 binds to the angiotensin-converting enzyme 2 receptor (ACE2) in the body. COVID-19 is known to exhibit a higher affinity for the ACE2 receptor than other virus groups. ACE2 receptor is frequently expressed in some lungs, kidneys, cardiovascular system, and gastrointestinal system. For this reason, these tissues are known to exhibit higher susceptibility to the disease than other systems [16,17].

In our study, we aimed to compare the percentage of positivity in fecal and urine samples of patients admitted and hospitalized with the suspicion of COVID-19, and also to compare the results of these patients using their biochemical and radiological findings.

Material and Methods

The current study was approved by the Ethical committee Medical Faculty of Kafkas University (date: 13.05.2020 no: 09).

Microbiological examination

In our study, urine and fecal samples were taken from a total of 50 patients hospitalized in COVID-19 wards. Routine biochemical tests, CT results, clinical symptoms, and all details of the respiratory tract RT-PCR tests were recorded, and the fecal and urine specimens were immediately stored in the -80˚ C refrigerator and prepared for the study. The presence of SARS-CoV2 RNA in urine and stool specimens was detected by qRT-PCR methods. Total RNA extraction was performed using QlAamp Viral RNA kit reagents (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ambiguous nucleotides (N) were omitted at the end of the process using AgPath-1Ds" One-Step RT-PCR Reagents (Thermo Fisher Scientific 120). Following this preliminary step, the readings for each sample were analyzed against the reference SARS-Cov2 (NC_045512.2) genome in the GenBank using the Map to Reference application in the Geneious program.

Radiological examination

As part of our Radiological Identification, samples were taken from patients hospitalized in COVID-19 wards, with CT scans performed simultaneously. General lung parenchyma CT findings to be investigated in COVID-19 virus pneumonia include organized pneumonia, diffuse alveolar damage, pneumonic consolidation, and infiltration combinations, which are generally quite nonspecific imaging findings. HlN1 and other types of influenza may present with similar appearance as adenovirus, cytomegalovirus pneumonia, atypical pneumonia, and inflammatory pneumonia. Accordingly, the severity of the disease should be classified according to conspicuous ground-glass images, segmental consolidation findings, and the intensity of involvement in CT. According to the report published by the "Radiological Society of North America (RSNA)" in March 2020, the classification of patients was grouped into four categories as "typical", "indeterminate", "atypical" and "negative". In our study, CT results of patients presenting with fecal and urine positivity were statistically evaluated together with respiratory-positive patients.

Biochemical examination

WBC, Neutrophil, Lymp, PLT, PCT, and RBC tests were performed on the BC-5380 Auto Hematology Analyzer (Mindray Bio-Medical Electronics CO.ltd.Shenzhen, China); BUN, CRP, LDH, AST, ALT, Total Bil, Direct Bil tests were performed on Cobas 8000 Modular Analyzer Series (Roche Diagnostics, Meinheim, Germany); and 25-OHD, Ferritin, and Troponin T

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values were measured on the Cobas 6000 Analyzer Series (Roche Diagnostics Meinheim, Germany). The results were saved on Excel program.

Molecular examination

Total RNA Isolation: Viral Gene-spinTM Viral DNA/RNA Extraction Kit was used for total RNA isolation. Fecal and urine samples stored at -80°C were transferred into lysis buffer without being refrozen-thawed and allowed to reach room temperature, then 20µL proteinase-K (20mg/mL) (SIGMA) was added to each sample and vortexed for 15 seconds. The vortexed samples were incubated at 55°C for 10 minutes, and the isolation process was completed following the steps in the commercial kit protocol. 2µL of the isolated samples were measured in a nanodrop spectrophotometer (Thermo Scientific, USA) to determine purity and density. 10µL of total RNA samples were also placed in 2% agarose gel electrophoresis at 90V for 60 minutes and the RNA band qualities were examined using the UV gel imaging system (Invitrogen, USA). The isolation processes of the samples were carried out by experienced personnel in the BSC Class II cabin, and all the consumables used were thrown into closed containers containing 10% bleach and disposed of in the medical waste.

Preparation of the complementary DNA library

Thermo Fisher Scientific High-Capacity cDNA Reverse Transcription Kit was used to prepare the cDNA library. For each sample, 20µL of total RNA isolate was included in the cDNA reactions, and the reactions were completed in accordance with the commercial kit protocol. All the steps carried out were completed on ice.

Real time PCR stages

Vi07918615-S1 (H5) (Thermo Fisher Scientific) TaqMan probes designed for the S1 gene (Spike1) of the SARS-CoV-2 virus were used in the PCR stages. 20µL Probe Mix (AMPIGENE® qPCR Probe Mix), 2µL probe, 8µL DEPC-treated water, and 10µL sample DNA (cDNA) were used to perform gene amplifications. Reaction conditions in the RT-PCR instrument (Applied Biosystems™ StepOnePlus™ Real-Time PCR System) were established as 95°C for 2 minutes (Initial denaturation and polymerase activation), 95°C for 5 seconds (Cyclic denaturation), and 60°C for 30 seconds (Cyclic attachment and elongation), in which the cyclic steps are repeated 45 times. A cDNA sample obtained from the total RNA sample isolated from the throat swab of a patient known to be infected with the SARS-CoV-2 virus was also included in each reaction plate as a positive control. As a negative control, a volume of DEPC-treated water equivalent to the amount of sample DNA used was added and included in the reactions. All PCR pre-steps were performed on ice.

Statistical analysis

The data obtained were transferred to IBM SPSS 26.0 software to create a data set. The Shapiro-Wilk normality test was applied initially to the obtained data. Parameters shown to be non-parametric by the normality test were assessed with the Mann-Whitney U test. The Pearson Chi-Square test was used to examine the relationship between grouped parameters with multi-well tables. A p value of <0.05 was considered statistically significant.

Results

While no positivity was detected in any of the 50 urine samples, the virus was traced in 15 fecal samples (Figure 1).

Again, in 4 of these patients, respiratory tract COVID-19 tests were negative while fecal samples turned out to be positive (Figure 2), which shows that viral shedding still continues in the stool of patients with negative respiratory tract findings. No positivity was found in 50 urine samples (Figure 1).

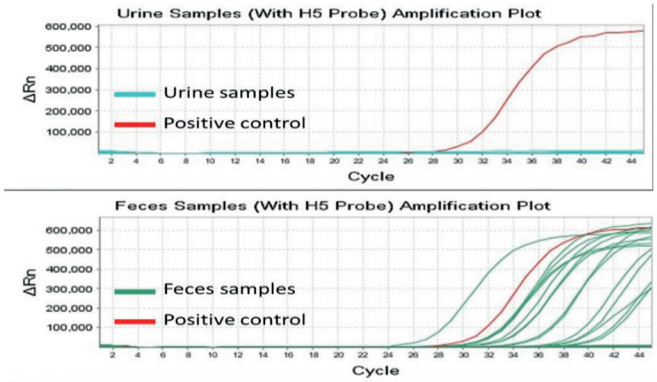

Figure 1. Positive urine and feces samples in PCR

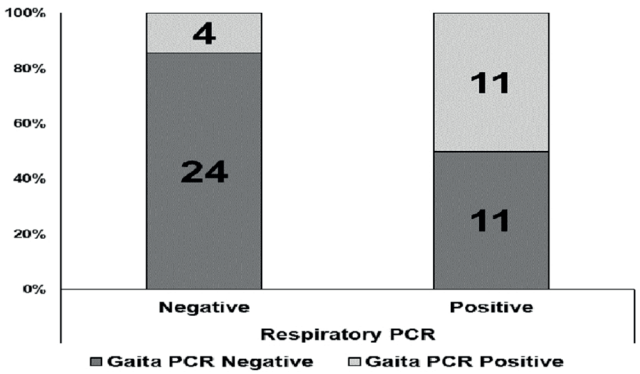

Figure 2. PCR positive and negative gaita numbers in respiratory positive and negative samples

The compliance between radiology findings and fecal and respiratory PCR specimens of the patients is shown in Table 1. Again, in Table 2, the correlation of biochemical parameters with fecal positive and negative patients is shown.

Comparison of patients' clinical symptoms and COVID-19 radiology classifications according to routine respiratory and fecal PCR test results is shown in Table 3.

Statistical analyses show that the mean age of fecal PCR-positive patients is much lower than that of negative patients (p=0.005).

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Table 1. Age, COVID-19 radiology classifications (CT results), clinical symptom classifications, and routine respiratory and fecal PCR test results of the patients included in the study

		Minimum	Median	Maximum	N (%)
Age [ab]p=0.398	**Female**	0	30.4[a]	76	21 (42)
	Male	0	29[b]	77	29 (58)
				N (%)	
Radiology COVID-19 classification (CT Results)	**Typical**	**Negative**		**Unspecified**	**Atypical**
	13 (27.7%)	18 (38.3)		12 (25.5)	4 (8.5)
				N (%)	
Clinical symptom	**Mild**	**Moderate**		**Severe**	
	32 (65.3%)	14 (28.6)		3 (6.1)	
				N (%)	
Routine respiratory PCR test results		**Negative**		**Positive**	
		25 (53.2)		22 (46.8)	
				N (%)	
Fecal PCR test results		**Negative**		**Positive**	
		35 (70)		15 (30)	

Table 2. Comparison of age, biochemistry, and hematological parameters of the patients according to the fecal PCR test results

	Fecal PCR negative			Fecal PCR positive			P
	Min.	Median	Max.	Min.	Median	Max.	
Age	0	47	77	0	16.9	76	**0.005**
CRP	1	10	140.09	0.3	16.5	252	0.764
Neutrophile	840	5455	16340	1140	1580	15600	0.273
Lymphocyte	450	1505	5680	320	1690	7240	0.649
LDH	189	253	563	166	249	519	0.990
AST	9.3	23.05	81	13.4	26.5	59.1	0.522
ALT	6.3	18.35	67	10	12.8	70.7	0.067
Troponin	3.3	10.9	58.7	6.3	16	24.6	0.404
WBC	5	8180	38170	3010	6840	17090	0.093
Prokalsitonin	0.021	0.057	1.69	0.035	0.06	1.71	0.946
Ferritin	12.6	141.5	1000.3	8.05	70.56	628	0.913
RBC	3450	5085	6280	4340	5110	5850	0.795
PLT	104	215	805	113	182	310	0.062
Bilirubin	0.1	0.385	3.01	0.12	0.42	0.84	0.756
BUN	14.1	31.55	215	11.6	35.5	86.3	0.625

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Table 3. Comparison of patients' clinical symptoms and COVID-19 radiology classifications (CT Results) according to routine respiratory and fecal PCR test results

		Fecal PCR Test Result			
		Negative	**Positive**	**Total**	
Clinical symptom	**Mild**	22	10	32	p=0.978
	Moderate	10	4	14	
	Severe	2	1	3	
		Routine Respiratory PCR Test Results			
		Negative	**Positive**	**Total**	
Clinical symptom	**Mild**	17	14	31	p=0.774
	Moderate	6	7	13	
	Severe	2	1	3	
		Fecal PCR Test Results			
		Negative	**Positive**	**Total**	
Radiology COVID-19 classification (CT results)	**Typical**	10	3	13	p=0.729
	Negative	12	6	18	
	Unspecified	9	3	12	
	Atypical	2	2	4	
		Routine Respiratory PCR Test Results			
		Negative	**Positive**	**Total**	
Radiology COVID-19 classification (CT results)	**Typical**	7	6	13	p=0.248
	Negative	8	10	18	
	Unspecified	6	6	12	
	Atypical	4	0	4	
		Fecal PCR Test Results			
		Negative	**Positive**	**Total**	
Routine respiratory PCR test results	**Negative**	22	3	25	**p=0.004**
	Positive	11	11	22	

Discussion

In our study, we aimed to compare the respiratory tract samples, which is the gold standard test procedure for COVID-19, with the fecal and urine system samples, as well as to determine the compatibility of the patients who were positive for COVID-19 in the respiratory tract and other samples with the biochemical and radiological parameters.

As is known, in the diagnosis of COVID-19, samples taken from the respiratory tract are routinely sent to the laboratory and the presence of the virus is examined by RT-PCR. Again, although the test result is negative in some patients, CT findings and biochemical tests in some patients indicate the presence of the virus. In addition, since 2019, the date when COVID-19 first emerged, it has become a pandemic with travels first to nearby cities, especially Wuhan, the city where it originated, and then to international countries. It is also known that at this time, the disease took over some cities and even urban quarantines were applied due to increasing cases around the world [18]. In light of all these data, considering the increase in urban cities, our aim was to question whether the disease could be transmitted not only by the respiratory tract but also by the fecal and urinary routes, and we detected the virus in the fecal samples of 15 of the 50 samples we collected at the beginning of the epidemic (Figure 1). Again, in 4 of these patients, respiratory tract COVID-19 tests were negative while fecal samples turned out to be positive (Figure 2). In this case, it shows that viral shedding still continues in the fecal samples of patients with negative PCR test

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results from respiratory tract samples. In our study, no significant difference was found between CT results and biochemical tests of fecal positive samples, and no urine positivity, which is around 1% worldwide, was detected (Figure 1) [19,20]. No statistical difference was found between age and gender (Mann-Whitney U; p=0.398) (Table 1).

While available studies yield a rate of 10% for positive stool, we found a rate of 7.5% in our study, which is compatible with the literature [19,20]. Again, a study conducted in Iran reported 7% positivity in the urinary system and 6% in fecal samples, whereas another study conducted on patients admitted with suspected pneumonia reported a rate of 35.7% [19,20].

In Türkiye, the number of studies on the subject is few. It was observed that most of these studies were in the form of compilation and information about the transmission of the virus in fecal samples. In one study, a rectal swap specimen was used instead of a direct fecal specimen [21,22].

While there was a significant difference between CT and biochemistry results of patients with positive respiratory tract samples, we did not find such a significant ratio between CT and biochemistry tests of patients with positive fecal samples. (Table 2, Table 3)

The virus is known to bind and shred, especially in the gastrointestinal tract, which is among the tissues where the ACE2 receptor is concentrated [16,17]. There are very few studies covering the etiology of patients and compatible CT and biochemistry results regarding the detection of COVID-19 in stool; therefore, the subject is of particular importance [23].

Studies show that the virus can be detected between day 3-7 and starts to disappear after day 15, but fecal shedding still persists [24]. In the study performed by Zhang et al., fecal samples were found positive in 10 (83.3%) of 12 patients, and respiratory samples were found positive in 14 (66.7%) of 21 patients. Another important point is that positivity can still be detected in the fecal samples of patients whose respiratory tract samples are negative on day 21, which shows that virus shedding persists through the fecal route even after the disease is overcome [25]. There are preliminary studies both abroad and in Türkiye, in which the proteins of the COVID-19 virus were detected in studies conducted with wastewater [26,27].

Conclusion

In conclusion, studies show that detection of COVID-19 in fecal or urine specimens can also be used in the future as an alternative method, especially in patients testing negative in respiratory specimens. Again, considering that viruses can survive in the external environment for a few days to a few weeks, in the light of our study and research, viral shedding in the stool is not to be underestimated, which raises the possibility that the positivity rates that rise in cities from time to time may spread from urban systems in such viral diseases.[28-30] Even today with a milder pandemic thanks to vaccines, when it comes to viruses such as COVID-19, the extent of viral shedding in fecal samples has not been addressed with sufficient studies yet, and prospective studies are needed on the subject. The next step of our study aims to sequence the fecal positive samples and to determine whether there is a significant difference between their prevalence according to the species.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

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Ethical Approval
The current study was approved by the Ethical committee Medical Faculty of Kafkas University (date: 13.05.2020 no: 09).

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